SCIENCE CHINA
Life Sciences

• RESEARCH PAPER •

December 2013 Vol.56 No.12: 1107–1112
doi: 10.1007/s11427-013-4570-5

TinII intron, an enhancer to affect the function of the cytoplasmic male sterility related gene *T* in *Brassica juncea*

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Received August 6, 2013; accepted September 9, 2013

The *T* gene, which was cloned from the mitochondria of tumorous stem mustard (*Brassica juncea* var. *tumida*), is a cytoplasmic male sterility (CMS)-related gene that can produce two transcripts, *T1170* and *T1243*. The latter is transcribed with the uncleaved intron *TinII*. In our previous study, transgenic *Arabidopsis thaliana* plants over-expressing the *T1243* transcript (OE-*T1243*) showed a severe male-sterile phenotype, whereas OE-*T1170* plants did not. However, the functional mechanism of the *T* gene in *B. Juncea* remained unknown. In this study, microscopic analyses of paraffin sections of anthers confirmed that OE-*T1243* plants did not produce normal pollen, whereas OE-*T1170* plants did. We analyzed the transcription of 15 anther development-related genes and found that transcript levels of *nozzle/sporocyteless* and *barely any meristem 1* and *2* were markedly lower in OE-*T1243* plants than those in wild type, while the transcript levels of these genes in OE-*T1170* plants were unchanged. To investigate the potential roles of *TinII*, we inserted the *TinII* sequence upstream of a minimal region (–60) of the cauliflower mosaic virus 35S promoter fused to the 5′ end of the β-glucuronidase (*GUS*) reporter gene. Analysis of the transgenic plants suggested that *TinII* acted as an enhancer to significantly increase *GUS* expression. The potential action mechanism is that the *TinII* intron acts as an enhancer to affect the function of the CMS-related gene *T*.

cytoplasmic male sterility (CMS), alternative splicing, enhancer, intron

Citation: Jin Z P, Wu L L, Cao J S, et al. *TinII* intron, an enhancer to affect the function of the cytoplasmic male sterility related gene *T* in *Brassica juncea*. Sci China Life Sci, 2013, 56: 1107−1112, doi: 10.1007/s11427-013-4570-5

An intron is any nucleotide sequence within a gene that is present in precursor mRNA but removed during processing into mature RNA. After intron splicing, the mRNA consists only of exons that are translated into the protein. Therefore, it has been assumed that introns have no biological function. With the development of molecular biology, many introns have been found to positively affect the expression of some genes, for example, *PAT1*, the histone *H3* gene, *ubq3*, *ubq10*, and *atpkl* in *Arabidopsis thaliana* [1–5]. Introns can enhance gene expression by 2-fold to 10-fold. However, not all introns enhance gene expression. Another important role of introns has been discovered in recent years; introns can be transcribed into microRNAs (miRNAs, which have important roles in regulating gene expression [6,7]. The precise mechanism by which introns regulate gene expression in plants is unclear. Introns differ from traditional enhancers since introns are thought to function via multiple mechanisms [8].

In recent years, there has been significant progress in research on anther and pollen development [9]. Many genes have been shown to be linked to anther and pollen development in *Arabidopsis*. Anther development begins with the

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induction of the transcription factor *NOZZLE/SPOROCYTELESS* (*NZZ/SPL*) by *AGAMOUS* (*AG*), an important MADS box transcription factor responsible for the formation of stamens and carpels in the third and fourth whorls of the wild-type (WT) flower [10]. *NZZ/SPL*, which shows homology to MADS-like transcription factors and plays an important role during archesporial division, is required for the initiation of sporogenesis during both stamen and carpel development. Pollen mother cells and the surrounding cell layers fail to form in the *nzz/spl* mutant. *NZZ/SPL* is an important link between the organ identity gene *AG* and downstream genes that play roles in anther development [11].

Some leucine-rich repeat receptor-like protein kinases (LRR-RLKs) play important roles in anther cell specification. Two of these, *BARELY ANY MERISTEM 1* (*BAM1*) and *BAM2*, act redundantly in cell fate specification in the anther. The *bam1* and *bam2* single mutants show no obvious phenotypic changes, strongly suggesting that these two genes are functionally redundant. Male *bam1bam2* double mutant plants fail to produce pollen and are, therefore, sterile [12]. BAM1 and BAM2 may form a positive-negative regulatory loop with NZZ/SPL to balance the number of sporogenous and somatic cells that form in the anther [11]. Some genes involved in anther development have been identified. The number of archesporial cells in the anther is strictly controlled by *EXCESS MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS* (*EMS1/EXS*), an LRR-RLK [13]. *TAPETAL DETERMINANT 1* (*TPD1*) is thought to act together with *EXS*. *tpd1* mutants show a phenotype similar to that of *exs*; they have extra meiocytes but lack tapetal and middle cell layers. The functionally redundant LRR-RLK genes *somatic embryogenesis receptor-like kinase 1* (*SERK1*)/*SERK2* may play a similar role in specifying somatic cell identity. The LRR receptor-like serine/threonine-protein kinase *ERECTA* (*ER*), *ERECTA Like 1* (*ERL1*), and *ERL2* all belong to the ER-family of LRR-RLKs. Together with *Arabidopsis* mitogen-activated protein kinases encoded by *MPK3*/*MPK6*, both ERL1 and ERL2 act redundantly in cell differentiation during early anther development. Mutants of *TPD1*, *SERK1/SERK2*, *ER*, *ERL1*, and *MPK3*/*MPK6* produce phenotypes similar to those of *exs* mutants. However, the *mpk3/mpk6* and *er/erl1/erl2* mutants are able to produce a tapetal layer [14,15]. *Glutaredoxin-C7* (*ROXY1*) and *Glutaredoxin-C8* (*ROXY2*), two *Arabidopsis*-specific CC-type glutaredoxin genes, are required for anther development. They act redundantly downstream of *NZZ/SPL* and play an important role in redox regulation in male gametogenesis [16].

Plant cytoplasmic male sterility (CMS) is a maternally inherited trait that is characterized by the failure to produce functional pollen. The mechanism underlying CMS had remained unclear for many years. Recently, however, a breakthrough was made in rice [17,18]. Members of the Cruciferae show considerable heterosis, so male-sterile lines are very useful for producing F1 hybrid cruciferae crops. The heterosis of Chinese tumorous stem mustard, a native vegetable, has been exploited to improve its quality and pest resistance. We have used distant hybridization and backcross procedures to breed a CMS line of stem mustard with superior economic traits [19]. The *T* gene cloned from this CMS line is a CMS-related gene that can produce two transcripts, *T1170* (AF298550) and *T1243* (JX040643). *T1243* is transcribed with the uncleaved intron *TinII*, which has some characteristics of a type II intron. In our previous study, we found that the *T* gene was expressed mainly in the *T1170* form in plants at the seedling stage, whereas the *T1243* form was prevalent in plants at the profuse flowering stage [20].

The potential roles of these two transcripts were studied in *Arabidopsis*. Transgenic plants over-expressing *T1243* transcripts (OE-*T1243*) showed a severe male-sterile phenotype characterized by almost fully collapsed siliques and anthers that appeared to be devoid of pollen. In contrast, the transgenic lines over-expressing *T1170* (OE-*T1170*) were normal [21]. The sequences of the alternative transcripts *T1170* and *T1243* were identical except for the uncleaved *TinII* intron in *T1243*. Therefore, it was assumed that *TinII* functions as a special *cis*-element to affect the expression of the *T* gene.

In this study, we compared WT, OE-*T1170*, and OE-*T1243* plants in terms of their anther morphology and the transcription patterns of genes related to anther development. To further explore the function of the *TinII* intron, we inserted it upstream of a minimal region (−60) of the cauliflower mosaic virus (CaMV) 35S promoter, which does not drive reporter gene expression in the absence of an enhancer. This construct was then fused to the 5′ end of the *β-glucuronidase* (*GUS*) reporter gene to analyze the role of *TinII* in transcriptional regulation.

1 Materials and methods

1.1 Flower and anther morphology

The growth conditions for *Arabidopsis* and the transformation method were described previously [21]. Open flowers were photographed under a bright-field microscope (Olympus SZ61, Tokyo, Japan). We prepared paraffin sections of anthers collected before blooming as described elsewhere [22]. The sections were stained in 0.5% hematoxylin at room temperature for 30 min and then observed under a Nikon Eclipse Ti microscope (Nikon, Tokyo, Japan) equipped with a Nikon DS-Fi1 camera. For these observations, we prepared sections of anthers from three independent transgenic lines.

1.2 Detection of transcripts of anther development-related genes

Flowers from 40-day-old WT, OE-*T1170*, and OE-*T1243* plants (T1 generation) were analyzed to detect transcript levels of anther development-related genes by RT-PCR as described previously [21]. The genes showing significant differential transcription were further analyzed by real-time PCR to confirm their transcription patterns. Analyses were performed using the BioRad Real-Time System CFX96TM C1000 Thermal Cycler (BioRad, Singapore). In the relative quantification analysis, the transcript level of each gene was normalized to that of *Tub2* as the reference gene. The results were analyzed using the $\Delta\Delta C_T$ method.

1.3 Transformation vector construction

The *GUS* gene expression construct was generated by inserting *TinII* upstream of a minimal (–60) 35S promoter that was fused to the 5′ end of the *GUS* gene. The *TinII* and (–60) 35S promoter-fused fragment (73+60 bp) was synthesized by Invitrogen (Shanghai, China). The sequence was as follows: GTGCGTTGTGGTATGCTATCTTCGAAGCTGGTACTGCACCTACAAACGCAGATAAAGATGTCGTGAAGGCTATCCCCACTATCCTTCGCAAGACCCTCCCTCTATATAAGGAAGTTCATTTCATTTGGAGAGA ((–60) 35S promoter underlined). The *TinII* and (–60) 35S promoters were synthesized at the same time. Each of these sequences was fused to the 5′ end of the *GUS* gene in pCAMBIA1301 as control constructs. *Eco*R I and *Nco* I sites were added at the 5′ and 3′ end, respectively, to the synthesized *TinII*-(–60)/*TinII*/(–60)35S fragments. After thorough digestion with *Eco*R I and *Nco* I, each of the fragments was ligated into the corresponding sites of pCAMBIA1301 that had been digested using the same enzymes, resulting in the recombinant plasmids pCAMBIA1301-*TinII*-(–60)-GUS, pCAMBIA1301-(–60)-GUS, and pCAMBIA1301-*TinII*-GUS (Figure 2A, a–c).

1.4 Analysis of GUS expression in transgenic plants

The T1 transgenic plants were screened on half-strength MS medium containing 25 μg mL^{-1} hygromycin. The presence of the construct in the hygromycin-resistant T1 plants was confirmed by PCR using the primers $P_{35S}1\times P_{35S}2$ (Table S1 in Supporting Information). Independent positive plants were self-pollinated to obtain T2 populations segregating from the homozygous lines. The genotype of the T3 plants was confirmed by hygromycin selection. The T3 seeds that did not segregate on MS plates containing hygromycin were identified as homozygous lines. The copy number of the transgene in transgenic plants was determined by Southern blotting using a *GUS* probe. To prepare the probe, we amplified the *GUS* gene fragment using the primers $P_{GUS}1\times P_{GUS}2$ (Table S1 in Supporting Information). The PCR products were purified before being labeled. Genomic DNA (10 μg) extracted by CTAB was digested overnight with *Eco*R V at 37°C before Southern blotting analyses were conducted. The DNA extracted from untransformed plants was used as a negative control [19]. For the detection of *GUS* transcripts by RT-PCR, total RNA was prepared from 100 mg tissue from 14-day-old seedlings of three T2 homozygous transgenic lines using Trizol® Reagent (Invitrogen, Carlsbad, CA, USA). Then, 5 μg RNA was used for reverse transcription using M-MLV reverse transcriptase as described by the manufacturer (Promega, Madison, WI, USA). Products were diluted and used as templates for PCR. The 986-bp *GUS* fragment was amplified by the primers $P_{GUS}1\times P_{GUS}2$. The transcript level of *Tub2* (for β-tubulin) as the internal control was determined using the primers *Tub*-1×*Tub*-2. All expression analyses were confirmed in three independent experiments.

2 Results

2.1 Morphology of *OE-T1170* and *OE-T1243* plants

In our previous study, about 83% (24 out of 29) of transgenic plants carrying the *T1243* construct exhibited male sterility, which was characterized by anthers devoid of pollen and almost fully collapsed siliques. However, OE-*T1170* plants did not show the male-sterile phenotype. The overall morphology and development were similar in OE-*T1170* plants and WT [21]. To further confirm the function of the *T* gene, we prepared paraffin sections of anthers and pollen to observe their morphology in the OE-*T1170* and OE-*T1243* plants. The OE-*T1243* plants displaying the severe male-sterile phenotype had no pollen grains in the anthers (Figure 1A, c and f). The anthers and pollen of OE-*T1170* were normal (Figure 1A, b and e) and were indistinguishable from those of WT (Figure 1A, a and d).

2.2 Transcript levels of anther development-related genes in OE-*T1170* and OE-*T1243* plants

T1243 can cause a severe male-sterile phenotype in transgenic plants. We speculated that *T1243* affects anther development-related genes, but *T1170* does not. To test this possibility, we used RT-PCR to evaluate the transcript levels of 15 anther development-related genes. Among them, only the mRNA level of *NZZ/SPL*, which played an important role during archesporial division, and the mRNA levels of *BAM1* and *BAM2*, which affected anther cell specification, showed marked decreases in *T1243* transgenic plants, compared with their respective levels in OE-*T1170* plants and WT (Figure 1B).

2.3 Effect of *TinII* on 60-bp *CaMV 35S* promoter

We generated pCAMBIA1301-*TinII*-(–60)-GUS/(–60)-

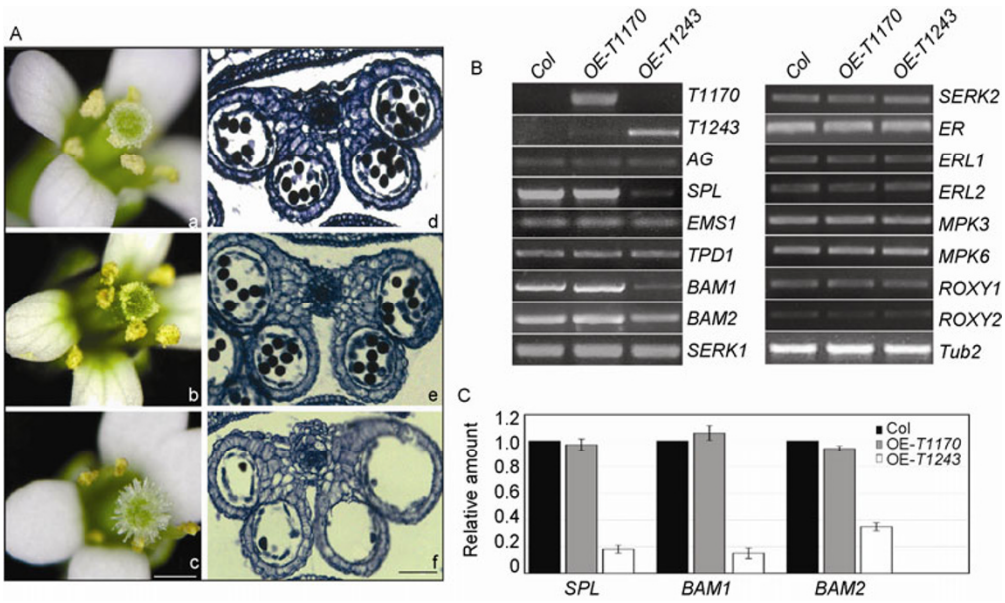

Figure 1 Comparison of WT, the OE-*T1170* and the OE-*T1243* plants. A, Comparison of flower and pollen morphology. a–c, Flowers of WT, OE-*T1170*, and OE-*T1243* plants, respectively; scale bar, 250 μm. d–f, Semi-thin sections of anthers from WT, OE-*T1170*, and OE-*T1243* plants, respectively; scale bar, 50 μm. B, Transcript levels of anther development-related genes in WT (lane 1), OE-*T1170* (lane 2), and OE-*T1243* plants (lane 3), relative to that of *TUB2* (internal control). Flowers from 40-day-old plants were used for this assay. Cycle numbers for PCR were 31–33 for anther development-related genes, and 35 for *Tub2*. C, Transcript levels of *SPL*, *BAM1*, and *BAM*2 were confirmed by real-time PCR. RNA amounts are relative to that of *Tub2*. Bars represent SD.

GUS/*TinII*-GUS (Figure 2A, a–c) constructs to verify the function of the *TinII* intron in *T1243* transcripts. The pCAMBIA1301-*TinII*-(−60)-GUS construct was generated by placing the *TinII* intron upstream of the minimal region (−60) of the CaMV 35S promoter (Figure 2A, a). The mini 35S promoter was fused to the *GUS* gene as a control (Figure 2A, b). The transcript level of *GUS* driven by *TinII* in the pCAMBIA1301-*TinII*-GUS construct (Figure 2A, c) was also detected. To investigate the integration of the construct into the genome of the homozygous transgenic plant, we analyzed three lines of each construct by Southern blotting, which showed that these plants contained single or multiple *T*-DNA inserts (Figure 2B).

We analyzed *GUS* transcript levels by RT-PCR to investigate the basis for the different transcriptions of pCAMBIA1301-*TinII*-(−60)-GUS, pCAMBIA1301-(−60)-GUS and pCAMBIA1301-*TinII*-GUS constructs. No *GUS* transcripts were detected in WT or pCAMBIA1301-*TinII*-GUS transgenic plants (Figure 2C, lanes 4–7) or two of the three pCAMBIA1301-(−60)-GUS transgenic plant lines (Figure 2C, lanes 8 and 10), and very low levels of *GUS* transcripts were detected in the third line (Figure 2C, lane 9). In contrast, *GUS* transcript levels were drastically increased in transgenic plants carrying pCAMBIA1301-*TinII*-(−60)-GUS (Figure 2C, lanes 1–3) compared with those in plants harboring the other two constructs, strongly suggesting that *TinII* acted as an enhancer in this cassette.

3 Discussion

Within introns, there are sequences encoding transcriptional regulatory elements, such as enhancers and repressors, which can regulate gene transcription. Introns can affect transcription by controlling DNA accessibility through modulating its position on the nucleosome. At the transcriptional level, splicing signals in an intron can further stimulate transcription by enhancing the initiation and processing activity of RNA polymerase II (Pol II) [8]. Previously, we reported that the *T* gene could produce *T1170* and *T1243* transcripts. The uncleaved *TinII* intron was present in transcript *T1243* and a 57-bp mini ORF was present in *TinII*. Over-expression experiments confirmed that *T1243* caused male sterility [21]. Our suggestion that *TinII* is a *cis*-element that acts as an enhancer was confirmed by the results of the present study. What is most puzzling is whether it is necessary to maintain this *cis*-element in the alternative transcript *T1243* and what the function of the small ORF in *TinII* is. We assume that the activity of the *TinII* enhancer is regulated by a negative *trans*-factor coupled with certain splicing-associated proteins. The negative regulatory factors would operate during cleavage of the *TinII* intron. Therefore, removal of the intron would lead to a loss of enhancement. In contrast, if the intron is retained, the splicing-associated factor and the negative *trans*-factor must be missing. It will be important to identify the *TinII*-binding protein in future research. To date, there have been no reports on the molecular mechanism of the *TinII* intron explained in this study. We attempted to obtain information about the function of intron *TinII* by bioinformatic analysis; however, we did not obtain conclusive results from online analyses. A BLAST search in the GenBank database revealed no homologs of *TinII* and no specific functional domain.

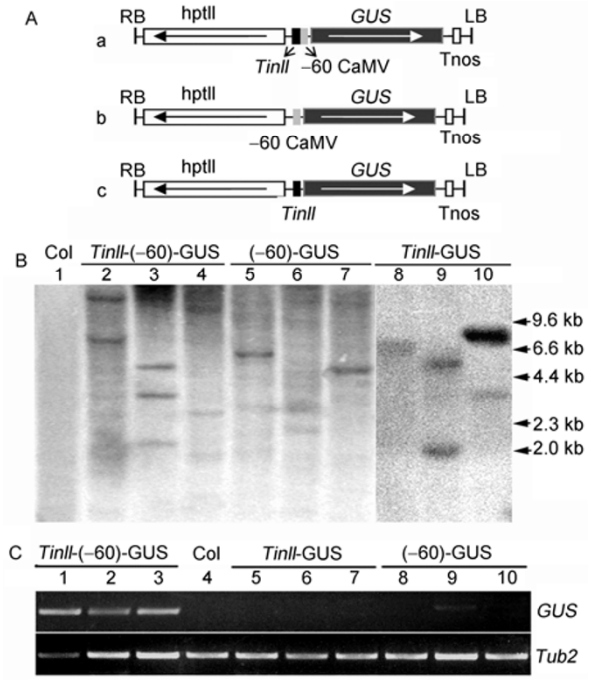

Figure 2 The function analysis of *TinII* intron. A, Schematic representation of constructs. Blank box with a white arrow represents *GUS* gene. Short black box and gray box represent *TinII* and −60 CaMV, respectively. a, *TinII*-(−60)-GUS construct in which intron *TinII* was linked to (−60) to drive *GUS* gene. b, (−60)-GUS construct. *GUS* gene was driven only by (−60) 35S. c, *TinII*-GUS construct. *GUS* gene was driven only by *TinII*. B, Southern blot analysis of transgenic plants harboring different constructs. 1, WT. 2–4, pCAMBIA1301-*TinII*-(−60)-GUS transgenic plants; 5–7, pCAMBIA1301-(−60)-GUS transgenic plants; 8–19, pCAMBIA1301-*TinII*-GUS plants. *GUS* coding fragment was used as probe. Leaves from 40-day-old plants were used in this assay. C, *GUS* transcript levels in T2 homozygous transgenic lines. Analysis of *GUS* transcript levels in WT, pCAMBIA1301-*TinII*-(−60)-GUS, pCAMBIA1301-(−60)-GUS, and pCAMBIA1301-*TinII*-GUS transgenic plants by RT-PCR, normalized to that of *TUB2* as internal control. 1–3, pCAMBIA1301-*TinII*-(−60)-GUS transgenic plants; 4, WT; 5–7, pCAMBIA1301-*TinII*-GUS transgenic plants; 8–10, pCAMBIA1301-*TinII*-GUS transgenic plants. For this assay, 14-day-old seedlings were used. Cycle numbers for PCR were 33 for *GUS* gene and 35 for *Tub2*.

In flowering plants, all mitochondrial group II introns are situated within protein-coding genes. More importantly, they share the consensus splice site 5′-GUGYG…AY-3′. *TinII* shows some of the characteristics of these consensus splice sites, but some aspects are not consistent with characteristics of group II introns. For example, a group II intron is typically 0.6–2.5 kb long, depending on whether an ORF is present. Typically, group II introns contain six conserved domains of secondary structure, D1–D6 [23]. However, there is a mini ORF of 54 bp in *TinII*, which itself is only 73 bp long and appears to be too short to act as a type II intron, even though group II introns without ORFs show a wide range of lengths. At present, it is unknown whether this small ORF encodes a *trans*-factor.

The 60-bp CaMV 35S promoter is frequently used as a tool for enhancer-trap studies because it avoids the potential effects of *cis*-elements, unlike the full-length 35S promoter sequence. This minimal promoter only drives expression of the reporter gene when there is a closely linked enhancer [24]. The expression patterns of the *GUS* reporter gene from *TinII*-(−60)-GUS, *TinII*-GUS, and two of the three (−60)-GUS lines were as expected, but only very weak expression was detected in one (−60)-GUS transgenic plant line (Figure 2C, lane 9). This may have been a result of the position of the transgene in the genome. In transgenic plants, exogenous genes are randomly integrated in different regions of the genome and enhancers that regulate neighboring genes can affect the expression pattern of the transgene. Therefore, there is potential for a unique gene expression pattern to exist at each site of integration.

This work was supported by the National Natural Science Foundation of China (31071809) to Pei YanXi.

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Supporting Information

Table S1 Accession number and primer of genes

The supporting information is available online at life.scichina.com and www.springerlink.com. The supporting materials are published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.